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VERIFICATION STATEMENT

The undersigned, Élise Regina Hendrick, verifies that she is knowledgeable of and fluent in both the German and English languages, and that the attached is a correct English translation of **DE 199 43 919 A1.**

ERH

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(Date)

METHOD FOR INCREASING THE YIELD OF RECOMBINANT PROTEINS IN MICROBIAL FERMENTATION PROCESSES

Industrial-scale production of recombinant proteins in bacteria takes place in fermenters. An increase in yield compared to laboratory-scale experiments done with flask shakers is achieved by increasing cell mass per volume. The batch-fed technique can achieve a high cell density. This is based on the growth-limiting addition of a nutrient source, whereby the carbon/energy source is generally limited (e.g. Riesenberg D. and Guthke, R., 1999, App. Microbiol. Biotechnol. 51, 422-430). For *E. coli* processes, this is normally glucose or glycerol. Alternatively, depending on the microorganism used and the process, however, other substrates are used, such as molasses, starch, peptone, lactose, methanol, and acetate. Highly concentrated feed solution can be added continuously, with the possibility of using various functions to define the addition of the substrate over a period of time; for example, addition may occur at a constant rate, or by exponentially increasing, or by linearly increasing and decreasing. Various functions are often combined within a single process. The addition of the substrate solution can also be regulated using other parameters. Dissolved oxygen (DO-stat), pH (pH-stat), or the concentrations of carbon dioxide and oxygen in the exhaust determined on line.

Standard vectors for gene expression are the plasmids, which, in addition to the replication origin, normally contain the DNA sequence that encodes for the desired protein (product gene), as well as a selection marker that serves to guarantee the stable preservation of the plasmid during culture growth. The expression of the product gene is normally controlled via regulatory sequences, particularly regulable promoters. Expression of the product gene is activated, for example, by chemical inductors (substrates, substrate analogues), changes in cultivation temperature or other culturing conditions (pH value, salt concentration, degree of substrate concentration). In particular, induction can also occur by changing the limiting substrate, or by induction of the tac-promoter with lactose and switching from glucose feeding to lactose feeding (Neubauer et al., 1992, Appl. Microbiol. Biotechnol. 36, 739-744).

Genes that provide the host cell with resistance to an antibiotic serve as selection markers for stable preservation of the plasmids in the host cells. Then, in the culture for the production of a recombinant protein, the corresponding antibiotic is normally

added to kill off or inhibit the growth of plasmid-free cells that do not carry the resistance gene. Commonly used resistance gene/antibiotic pairs are β-lactamase/ampicillin, chloramphenicol-acetyltransferase / chloramphenicol, tetracycline resistance (tet)-operon/tetracycline, and kanamycin resistance gene / kanamycin.

Some of these resistance systems have the disadvantage that the antibiotic is deactivated by the resistance gene, as with ampicillin and chloramphenicol (e.g. Kemp G.W. and Britz M.L., 1987 Biotechnol. Techniques 1, 157-162). The consequence of this deactivation is that there is no obstacle to multiplication of plasmid-free cells in the culture. In addition, the proteins that mediate resistance can be released into the medium in the preparatory culture, accelerating the breakdown of the antibiotic. In these cases, the proportion of plasmid-free cells in the total culture can be increased. Moreover, no antibiotics are used in a large number of industrial processes for cost reasons or due to the additional expense incident to the subsequent cleaning, in which the remaining traces of the antibiotic or its deactivated form must be removed. A certain proportion of plasmid-free cells appears in such processes, as well.

While plasmid-free cells often only have a small growth advantage in the growth phase, in many cases, after product formation has begun, reduction in the growth rate of plasmid-containing, producing cells occurs, thus causing the culture to be overgrown by the plasmid-free cell population. The accumulation of plasmid-free cells has the disadvantage of reducing the relative proportion of the product in the total cell mass, and, depending on the decomposition and cleaning methods chosen, making these postfermentation steps more difficult.

When constructing the vector, it is possible to limit these adverse effects, e.g. through selection of the resistance gene, the use of alternative, antibiotic-independent stabilization systems (Molin and Gerdes, WO84/01172), or by using modified antibiotics that break down more slowly; but the problematic resistances are still used. Moreover, none of the alternative systems is infinitely stable; stability can only be maintained for a certain time span.

The invention disclosed in Patent Claim 1 is based on the problem of suppressing the overgrowth of plasmid-free cells after induction of recombinant product synthesis in batch-fed fermentations, particularly in industrial applications.

The characteristics listed in Patent Claim 1 solve this problem by increasing / decreasing the concentration of the carbon/energy source in a cyclically oscillating pattern. This is achieved by changing the rate of addition of the feed solution that contains the carbon/energy source, e.g. by corresponding programming of the pump that doses the feed solution. This leads to sequential phases, in which the cells either have a limited amount of substrate available to them or none at all.

While such cyclical processes are occasionally used, e.g., in order to simulate mixing problems occurring in large-scale industrial production in small femrenters (Neubauer et al., 1995, J. Biotechnol. 43, 195-204) or to increase metabolic rates (Qian, 1998, Patent No. CN 1177007), the general view has heretofore been that oscillations have adverse effects on product formation in recombinant processes. Surprisingly, however, targeted oscillations can have a positive impact on the process.

The advantage of this method, which is principally applicable to all recombinant growth-limited processes in which the formation of the recombinant product is induced under carbon limitation, is that it is not necessary to add any further substances to the fermentation medium, that it is independent of the expression system used, and that it has no adverse effects on product formation. This procedure is particularly suited for batch-fed processes, in which a sugar, such as glucose, lactose, arabinose, or galactose, or other organic carbon sources, such as methanol, glycerol, molasses, or starch as a limiting nutrient are added to the culture. The procedure is independent of the cultivation medium, and can be used for cultivation on mineral salt medium as well as complex media.

Short cycle times of app. 1 min (30 sec feeding, 30 sec pause) in particular are advantageous. Extension of the cycle time, on the other hand, adversely affects product formation.

This method is not limited to *Escherichia coli* as host organism; rather, it can be used with all microorganisms, such as *Bacillus subtilis, Saccharomyces cerevisiae,* or *Pichia pastoris*, which are cultivated using carbon-limited fed batch. It is also independent of the induction system. However, it is particularly advantageous when using the tac-promoter.

The procedure is particularly advantageous when the expression of the gene product is strongly induced and growth of the producing cells is adversely influenced compared to a noninduced culture. In addition, this procedure is advantageous in processes in which the production phase is particularly long, e.g. in the periplasmatic expression of recombinant proteins or when the product formation phase is connected with a shift in temperature.

Exemplary Embodiment

Strain and Plasmids

Escherichia coli K-12 RB791 (F^-, IN (rmD-rmE)1,λ^-,lacI^{q}L$_g$; *E. coli* Stock Center, New Haven, USA) was used as the host. This strain was transformed with the plasmid pKK177glucC (Kopetzki et al., 1989a), in which the α-glucosidase gene from *Saccharomyces cerevisiae* is under the control of the tac-promoter. The plasmid contains the β-lactamase gene as its selection marker. Additionally, a second system was used, in which the plasmid pUBS520 (Brinkmann et al., 1989), which contains the *dnaY* gene (Minor-tRNA *argU,* AGA/AGG), was transformed in addition to the plasmid pKK177glucC.

Cultivation Medium and Fermentation Conditions

Glucose-ammonium-mineral salt medium (Teich et al., 1998, J. Biotechnol. 64, 197-210) was used for all cultivations. The initial concentration of glucose was 5 g l^{-1}. The feed solution contained 200 g glucose kg^{-1} and all components of the cultivation medium in the corresponding concentrations (Exception: $(NH_4)_2SO_4$ 2.0 g l^{-1}) and 10

ml l^{-1} of the trace element solution (Holme et al., 1970), but no $MgSO_4$. This was added during cultivation at 10ml of a 1 M $MgSO_4$ solution with OD_{500}=9. Ampicillin (100 mg l^{-1}) and kanamycin (10 mg l^{-1}) were added both to the preparatory cultures and the fermentation medium. Polypropylene glycol 2000 (50 %) was used as an anti-foam agent.

Shake cultures on fermentation mineral salt medium, grown at 37°C, were used as the fermentation inoculum. All fermentations were carried out in 6 l Biostat ED Bioreactors with an initial volume of 4 L and a temperature of 35°C. The cultures were started as a batch culture. In this phase, the aeration rate and the stirring were regulated in a cascade mode in order to maintain a DOT of at least 20%. At the end of the batch phase, the DOT control was deactivated and the aeration rate and stirring speed were set at 2 vvm or 800 rpm. The pH value was regulated at 7.0 using a 25% ammonia solution. At the end of the batch phase, at a cell density of app. 2 g DCW l^{-1} (OD_{500}=9), the feeding pump was started at a constant rate of 53.2 g h^{-1} (2.6 g glucose l^{-1} h^{-1}). The total amount of glucose added was the same in all cultivations, independent of the feed mode. Three different feeding strategies were tested: (A) continuous feeding (controlled cultivation), (B) intermittent feeding with a cycle of 1 minute (30 seconds on, 30 seconds off), (C) intermittent feeding with a cycle of 4 minutes (2 minutes on, 2 minutes off). The expression of the α-glucosidase gene was induced after adding 1 mM IPTG 3 h after feeding was started, and product formation was followed over a time span of app. 20 h after induction.

Analytical Methods

Cell growth was followed by measuring the optical density at 500 nm (OD_{500}). The microscopic cell count was further determined in a counting chamber (0.02 mm depth), and the dry cell weight (DCW) was determined (see Teich et al. 1998, J. Biotechnol. 64, 197-210). The number of colony forming units (cfu) was determined by outcropping diluted samples on nutrient agar plates that were incubated for at least 3 days. Plasmid stability was then determined by overstamping these plates on selective agar with the replica plating technique. The relationship between DCW, OD_{500}, and cell count was characterized as follows: 1g/l DCW corresponds to an

OD_{500} of 4.5±0.1 and a cell count of $1.8x10^9$ ml^{-1}. The glucose concentration was determined using a commercial enzyme kit.

The α-glucosidase concentration was determined after separating total cell samples in SDS gel (5% collection gel, 7% separation gel). Expression was carried out by scanning the product strip and quantification compared with a product standard placed in the gel in various concentrations.

Results

E. coli RB791 pKK177glucC and *E. coli* RB791 pKK177glucC pUBS520 were cultivated in an agitation reactor using glucose-limited fed batch. After the first batch phase, constant feeding was started and, three hours after the start of feeding, the expression of the α-glucosidase gene was induced by adding 1 mM IPTG. After induction, there is an increase in the α-glucosidase concentration, whereby the specific concentration of the enzyme per cell reaches its maximum approximately 5 h after induction, and begins to reduce in longer cultivation (see Fig. 1c). The reduction of the specific concentration of α-glucosidase is due to the overgrowth of the culture with plasmid-free cells. These have an enormous growth advantage after induction, as the production of α-glucosidase adversely affects growth and also causes an inhibition of glucose uptake in the producing cells. This leads to accumulation of glucose in the culture medium. Cells present in the culture that do not contain the product gene are not influenced by the inductor IPTG, but rather continue to grow without limitation due to the high availability of glucose.

However, overgrowth of the culture by the plasmid-free cell population can be prevented depending on pulse duration (see Fig. 1d). This positive effect on the suppression of plasmid-free cells was not only obvious in the strongly expressing system shown in Fig. 1, but also in the weak expression of α-glucosidase in the *E. coli* RB791 pKK177glucC system (Fig. 2, table 1). Moreover, pulse feeding had a slight positive influence on the synthesis rate in both cases after induction, and, in the first case, also on the stability of the product, more than 90% of which was present in the form of inclusion bodies.

Table 1: Productivity and Overgrowth by Plasmid-Free Cells in Glucose-Limited Fed-Batch Cultures of *E. coli* RB791 pKK177glucC with and without PUBS520

Type of substrate added during fed-batch fermentation	α-glucosidase yield [mg/g biomass]		Plasmid-free cells [% of total population]	
	3 h post induction	20 h post induction	3 h post induction	20 h post induction
RB791 pKK177glucC pUBS520				
Constant feeding	37	30	2	72
Cycle 1 min	38	24	1	16
Cycle 4 min	37	6	2.5	60
RB791 pKK177glucC pUBS				
Constant feeding	10	9	10	10
Cycle 1 min	14	10	0.3	2.7
Cycle 4min	6	4.6	15	6.7

The figures show:

Fig. 1: Fed-batch fermentations with *E. coli* RB791 pKK177glucC pUBS520 with induction by 1 mM IPTG. Comparison of continuous addition of glucose substrate solution (a-c; open symbols: without induction; filled symbols: with induction) with cyclic addition (d-f) of the same solution (σ: cycle of 1 min; ∇ cycle of 4 min). (a,d) cell mass (DCW), (b,e) glucose concentration, (c,f) product formation (mg α-glucosidase / g cell dry weight). The data shown represent a characteristic fermentation of 2 experiments performed for continuous addition and 1 experiment each for cyclic addition. Starting time for the addition of substrate solution (------), induction with IPTG took place 3 h after feeding start (. . . . . .).

Fig. 2: Fed-batch fermentations with *E. coli* RB791 pKK177glucC with induction by 1 mM IPTG. Comparison of continuous addition of the glucose substrate solution (a-c; open symbol: without induction; filled symbol: with induction) with cyclic addition (d-f) of the same solution (σ: cycle of 1 min; ∇: cycle of 4 min). (a,d) cell mass (DCW), (b,e) glucose concentration, (c,d) product formation (mg α-glucosidase / g cell dry weight). For further explanations, see Fig. 1.